

Apoptosis in sepsis

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Sepsis demonstrates a marked dysregulation of the immune system in its ability to fight infection. Previous models have focused on the mechanisms which upregulate and sustain the heightened immune response without addressing the role of down-regulation effectors. Attention has been drawn to these down-regulating mechanisms and their precise role in the pathophysiology of sepsis. Apoptosis is an evolutionarily conserved, energy-dependent mode of cell death requiring the initiation and regulation of complex genetic programs. It is the body's main method of getting rid of cells which are in excess, damaged, or no longer needed in a controlled manner. The role of this cellular phenomenon in physiology and pathophysiology has been the

subject of intense scrutiny over the last decade. Much work has demonstrated that dysregulation of apoptosis does occur in immune and nonimmune cells in *in vitro* and *in vivo* models of sepsis. The difficulty has been in tying the phenomenology of apoptosis into the pathophysiology of sepsis. Further work is needed to make these connections to elucidate rational approaches for clinical applications of immunomodulation in sepsis. (Crit Care Med 2000; 28[Suppl.]:N105-N113)

KEY WORDS: apoptosis; sepsis; systemic inflammatory response syndrome; neutrophil; lymphocyte; endothelial cell; hepatocyte; nitric oxide; inducible nitric oxide synthase

Homeostasis is defined as the maintenance of the internal milieu. Physiologically, it refers to all of the interdependent, multisystem, multiorgan mechanisms an organism employs to function normally. Sepsis marks a dramatic dysregulation of the immune system in its ability to maintain homeostasis in the face of infection by microorganisms (1). Historically, sepsis was thought to represent the appropriate but inadequate mobilization of host defenses against an overwhelmingly virulent infection (2). Two key observations changed our thinking of the pathophysiology of sepsis. The first observation was that the phenotype of system-wide, uncontrolled inflammation leading to subsequent multiple organ failure (MOF) could occur from trauma, hemorrhage, or transfusion in the absence of infection (3). The second discovery was that soluble mediators elaborated from stimulated leukocytes could also replicate the phenotype of system-wide inflammation (systemic inflammatory response syndrome [SIRS]) and MOF (4).

The model of sepsis and SIRS that had emerged was essentially one of persistent and uncontrolled inflammation that ultimately caused system-wide effects, shock, and end organ failure. The theory of rampant uncontrolled inflammation, combined with the discovery of small potent proteins secreted from immune cells, lent itself to the intense study of proinflammatory cytokines and the Herculean effort to systematically catalogue this complex aspect of immune physiology. Enthusiasm for this line of research saw its peak in clinical trials aimed at blocking the early mediators of the proinflammatory response in sepsis (5-9). The results of these studies have consistently failed to show any benefit in the immunomodulation of proinflammatory mediators. This has led to a reevaluation of the model of sepsis and dysregulated systemic inflammation.

Homeostasis requires compensatory mechanisms to maintain the internal milieu. The immune system has evolved proinflammatory and anti-inflammatory programs to deal with invasion by foreign agents. This is seen clinically in that the body can mount an overwhelming overreaction or underreaction to injury or infection (10). Importantly, dysregulation in the execution of either set of programs may result in failure to reestablish homeostasis. Mechanisms for marshaling an organism's resources to combat infection are necessarily interrelated with sub-

sequent down-regulation of these responses once the infectious agent has been killed (Fig. 1). Less attention had been directed in the study of mechanisms for down-regulating the immune response. In addition, focus on extracellular mediators had left the role of intracellular responses to injury and infection largely unknown. Most recently, these two areas have been the focus of scrutiny in sepsis research.

It has become increasingly clear that apoptosis, or cellular suicide, is one of the most important, biologically conserved mechanisms for multicellular organisms to respond to external injury (11). Recent work has revealed the integral nature of apoptosis in the regulation of immune cells. The mechanisms of initiation, second signaling, and effector function are gradually being elucidated. The complexity of this cellular process is similar in its murkiness to the early work describing the physiology of cytokines, immune cells, and end organ effects. What has emerged is a tangle of tantalizing pieces to a puzzle, which focuses on some of the most basic aspects of physiology.

It is also clear that apoptosis plays a fundamental role in the function of the "normal" immune response (12). Less clear is the role that apoptosis and its dysregulation may play in pathologic immune states like sepsis, autoimmune disease, or immunosuppressed states.

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Linking the precise role of this process to the outcome of immune responses is difficult. The potential role of apoptosis and its dysregulation in the pathogenesis of sepsis has focused on three basic questions. The first is whether impairment of apoptosis as a method of down-regulating proinflammatory cells causally results in inappropriate persistence of inflammation under septic conditions. The second question is whether increased apoptosis of immune effector cells results in immunosuppression and increased susceptibility to overwhelming infection. Finally, do environmental conditions during sepsis and SIRS result in the apoptosis of end organ parenchymal cells and causally result in end organ failure (Table 1)? Unfortunately, many of the pieces of data

needed to connect the descriptive information available to the ultimate role of apoptosis in the pathophysiology and outcome of infection are not known.

We will begin with a review of the basic molecular events involved with apoptosis. A comparison with the process of necrosis will illustrate the difference between these two processes of cell death and the potential implication in the pathogenesis of inflammation. The next section of the review will describe the role of apoptosis in the "normal" regulation of neutrophils and lymphocytes. This will be followed with a review of the available information examining the dysregulation of immune cell apoptosis in sepsis. Work from our group and others will illustrate the role of apoptosis in paren-

chymal cells with special attention to the role of nitric oxide in the physiology of apoptosis in endothelial cells and hepatocytes. Finally, we will review the studies which have tried to tie the dysregulation of apoptosis seen in sepsis with end organ morbidity and overall mortality. To conclude, we will attempt to synthesize the available information into a framework to describe the role of apoptosis in the pathophysiology of sepsis.

APOPTOSIS

Apoptosis refers to the morphologic alterations exhibited by "actively" dying cells. It includes a typical set of changes including cell shrinkage, cleaving of nuclear deoxyribonucleic acid (DNA), chromatin condensation, and membrane blebbing (13). In 1971, Kerr first described this type of cell death as "shrinkage necrosis" to draw a clear distinction between the types of cell death that occur during animal development, tissue homeostasis, and pathologic states (14, 15). Teleologically, apoptosis allows a multicellular organism the ability to direct resources to control cell numbers. Homeostasis necessitates dedicated mechanisms not only for cell proliferation but also cell death. Practically the body must be able to rid itself of cells that are no longer needed, that have been produced in excess, that have developed improperly, or that have sustained irreparable damage. The term "apoptosis" is now generally used to describe the evolutionarily preserved pathway of biochemical and molecular events leading to cell demise (16, 17).

The process of active cell death can be divided into three phases: a) initiation; b) effector; and c) degradation (Fig. 2). Numerous stimuli can initiate apoptosis including physiologic activators like tumor necrosis factor, Fas ligand and calcium; cell damage-related inducers like heat shock, reactive oxygen species, cytolytic T cells, and p53; therapy-associated factors like γ irradiation and chemotherapeutic agents; and toxins like ethanol and amyloid protein (18, 19). Some, but not all, of these damage stimuli initiate cell signals via a family of homologous receptors known as death receptors of which the tumor necrosis factor (TNF) receptor is the most well recognized. These proteins have an extracellular binding site rich in cysteine residues and a highly conserved cytosolic death domain which enables the cell's apoptotic machinery. These receptors

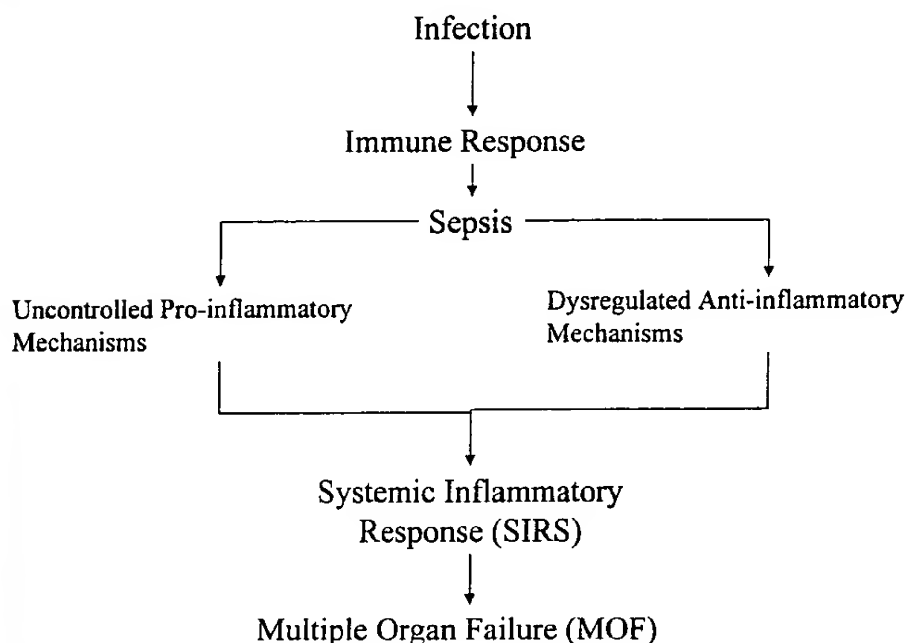


Figure 1. Pathophysiology of sepsis.

Table 1. Apoptosis in the pathophysiology of sepsis

Observation	Hypothesis
Delayed neutrophil apoptosis	Beneficial Enhanced function Prolonged function Detrimental Prolonged elaboration of toxic metabolites May result in neutrophil necrosis
Increased lymphocyte apoptosis	Beneficial Decreased autoreactive clones Decrease in effectors which can perpetuate inflammation Detrimental Immunosuppressive
Parenchymal apoptosis	Beneficial Decreased burden of dying or senescent cells No bystander inflammation Detrimental Decreased functional capacity of the organ

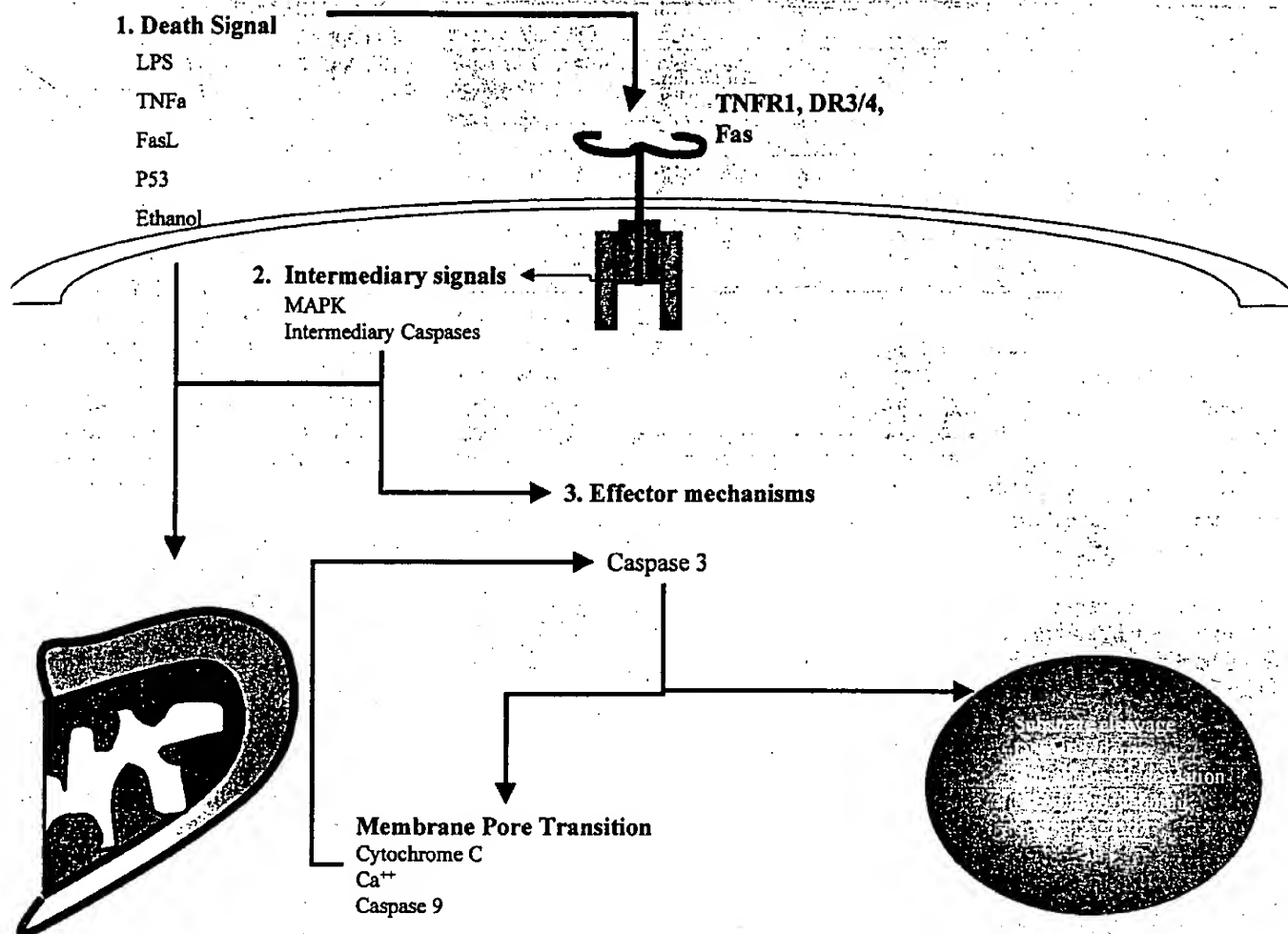


Figure 2. Schematic cartoon of the basic machinery of apoptosis. Signals activate death programs via specialized receptors or by directly stimulating effectors. Secondary signals work through multiple pathways to activate apoptosis effector mechanisms like mitochondrial membrane pore transition and activation of caspase. Effector mechanisms initiate a number of degradative changes including substrate cleavage, DNA laddering, chromatin condensation and membrane blebbing. *LPS*, lipopolysaccharide; *TNFα*, tumor necrosis factor-α; *MAPK*, mitogen-activated protein kinase.

work through a variety of intermediary signaling molecules including death effector domains and caspase recruitment domains to initiate secondary signals which, in turn, regulate the effector arm of the cells apoptotic machinery (20). The most prominent of the secondary signaling pathways includes that of the mitogen-activated protein kinases and of the intermediary caspase molecules (21, 22).

During the effector phase, many of the pathways converge. There appear to be two main series of intracellular events that comprise committed steps by the cell toward apoptosis. One is mitochondrial membrane permeability transition. In response to proapoptotic intermediary signals, the inner mitochondrial membrane opens a pore which allows the equilibration of molecules up to 1500 daltons in mass. This results in the loss of the

mitochondrial membrane potential, flux of Ca⁺⁺, and redistribution of cytochrome c into the cytosol. Importantly, these events lead to the activation of caspase 9 and subsequent downstream effectors like caspase 3 (11). This also appears to be sufficient in initiating the degradative arm of the apoptotic machinery.

The second main committed apoptotic event is the activation of caspase 3 (23). Caspase proteins are named for their cysteine containing catalytic site that recognizes and cleaves protein substrates after specific aspartic acid residues. The significance of these enzymes was first noted in their homology with programmed cell death genes first described in the embryologic development of *Caenorhabditis elegans* (24). In the nematode, there are three main proteins, ced-3, ced-4, and ced-9, which can initiate or inhibit apo-

ptosis when overexpressed. The respective mammalian analogues are caspase 3 which is proapoptotic, APAF-1 which serves a chaperone function, and the Bcl family which contains proapoptotic and anti-apoptotic members (25-27).

Activation of caspase 3 in response to proapoptotic signals results in several proteolytic events, which irrevocably lead to cell death. One event is a feedback effect on the mitochondria for further pore formation. A second event is translocation of caspase 3 into the nucleus which results in substrate cleavage, DNA breakdown, and protein modification.

The degradative phase results in the morphologic appearance that is distinctive of apoptosis. These include cleavage of nuclear DNA into oligonucleosomal fragments of multiples of 200 kbp, condensation of chromatin, cytosolic shrinkage,

Table 2. Modes of cell death

Apoptosis	Necrosis
Cell shrinkage	Cell swelling
Rapid phagocytosis	Organelle swelling
Noninflammatory	Membrane rupture with spilling of cytoplasmic contents
Energy dependent	Inflammatory
Regulated	Bystander effect
Ordered breakdown of nuclear DNA and organelles	No energy requirements
Cytoskeletal rearrangement	Random DNA breakdown
Flipping of phosphatidyl serine residues to the outer leaflet of the plasma membrane	

DNA, deoxyribonucleic acid.

and flipping of phosphatidyl serine moieties to the outer leaf of the plasma membrane (28). There is no widespread swelling and decomposition of intracellular organelles as seen in cell death by necrosis. The end result is what is seen on light microscopy as the shriveled apoptotic body. The distinctive phosphatidyl serine residues are rapidly recognized and allow the efficient removal of these dead cells by phagocytes (29). This serves two beneficial purposes: the first is the ability to reutilize portions of the apoptotic body for future use; the second is scavenging of the area before cytosolic molecules can leak out and induce an inflammatory response.

Apoptosis Versus Necrosis

The above discussion characterizes apoptosis as a defined, regulated, mechanistic mode of cell death. The evolution of this intricate and complex genetic program highlights that this mode of cell death does not occur by accident. Apoptosis is rigidly controlled and requires energy in the form of ATP to carry out all of its attendant requirements. It is not accompanied by an acute inflammatory response and does not necessarily induce an identical cascade of cellular events in nearby tissues of similar or different histology.

This is in contradistinction the usual type of cell death with which we are familiar, necrosis (Table 2). Necrosis involves cytoplasmic, organelle, and plasma membrane swelling. Necrosis involves random DNA breakdown, not like the ordered packaging into oligonucleosomes seen in apoptosis (30). Swelling of cells dying by necrosis inevitably leads to disruption of cellular compartments with leaking of intracellular debris into the local environment. Disordered and inefficient scavenging of this debris allows for

the up-regulation of a local inflammatory response and a potential cascade of similar injurious effects on nearby cells (31). Death by necrosis does not involve the dedicated mobilization of cellular machinery and, as such, has no specific energy requirements. In its purest sense, necrosis is the exact opposite of apoptosis as a cellular response to injury.

The complexity of life, however, would not and does not allow for such rigid categorization to describe the variety of methods that a cell can respond to injury. A number of stress response programs have been described to account for changes in cell phenotype after injury. These stress responses include the heat shock response, the acute-phase response, the hypoxia/ischemia response, and responses to oxidative stress. Depending on the severity of the injury stimulus interpreted in the context of the intracellular milieu, the cell executes a defined series of genetic programs to protect itself.

Mild or moderate stimuli initiate stress response programs that serve to marshal cellular resources to "weather the storm." More severe stimuli can be envisioned as signaling an unsurvivable insult resulting in the initiation of death programs of apoptosis. Finally, at the furthest end of the continuum, the most severe injuries would leave the cell unable to protect it and result in death by necrosis. The phenotype of cell death comprises a continuum depending on the relative severity of the injury stimulus and the relative "hardiness" of the cell that is injured.

Immune Cell Apoptosis in the "Normal" Immune Response and in Sepsis. Over the last 10 yrs, it has been shown that apoptosis plays an important role in the normal regulation of maturation, differentiation, proliferation of immune

cells, and termination of immune responses (12). In the next section, the role of apoptosis in the regulation of neutrophils and T lymphocytes will be explored because of the importance that these cells play in normal responses to infection and models of sepsis.

NEUTROPHILS

Neutrophils are the first immune cells to migrate to sites of inflammation (32). Their primary functions include phagocytosis of bacteria, elaboration of oxidative and nonoxidative degradative enzymes, and the elaboration of chemotactic factors to recruit other inflammatory cells (32). Most neutrophils that migrate from the vasculature to sites of injury die there (29). Unlike lymphocytes which can transform into memory cells, migrate to secondary lymphoid tissue and take on secondary surveillance function, the granulocyte has a finite lifespan; once activated, the granulocyte will die after a specified period of time.

Apoptosis of neutrophils may be one of the mechanisms of limiting tissue injury at sites of inflammation. It has been known for over a hundred years that intact neutrophils are phagocytosed by monocytes at inflammatory sites (29). It was first shown that phagocytosis by macrophages is the major mode of neutrophil clearance after experimental peritonitis (33). This observation has been corroborated in inflammation models of the joint, lung, gut, and kidney (34-37).

It was noted that neutrophils spontaneously undergo apoptosis in culture. Macrophages would not ingest fresh neutrophils but would eat up older ones. Neutrophils aged more than a day, however, would begin to necrose as seen in the appearance of cytosolic proteins like myeloperoxidase in culture supernatants. By centrifugal elution techniques, it was shown that apoptosis was the change in the senescent neutrophil which initiated phagocytosis (36).

Several lines of *in vitro* evidence indicate that apoptosis of neutrophils may limit injury in inflammation. First of all, apoptotic neutrophils have suppressed respiratory burst activity (38). Apoptotic neutrophils are unable to degranulate and lose important activation-associated ligand receptors like intercellular adhesion molecule-1 (ICAM-1) (39). Phagocytosis of apoptotic neutrophils inhibits the release of proinflammatory cytokines from monocytes (40). Ingestion of necrotic

polymorphonuclear leukocytes results in cytokine release.

Lipopolysaccharide (LPS), a neutrophil phagocytosis of neutrophils to project a neutrophilic activity, situation complete death signal function sepsis is a sign of tissue injury, hypothesis apoptosis of neutrophils to with the inflammation of bystander

Neutrophils in Sepsis

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polymorphonuclear cells, however, results in an intense release of the molecules.

Lipopolysaccharide, granulocyte-monocyte colony-stimulating factor (GM-CSF), and glucocorticoids can inhibit neutrophil apoptosis (41). Alternatively, phagocytosis of *Escherichia coli* and ligation of the Fas receptor can speed up neutrophil apoptosis (42). It is appealing to project a model in which apoptosis of neutrophils is delayed when inflammatory signals indicate a need for longer activity. It is also attractive to envision a situation that once the neutrophil has completed its function, it receives its death signal. One hypothesis linking dysfunction of neutrophil apoptosis with sepsis is that delays in neutrophil apoptosis may allow for the prolonged elaboration of tissue destructive and proinflammatory chemotactic factors. A second hypothesis is that delays in neutrophil apoptosis may allow senescent neutrophils to progress to death by necrosis with the detrimental leakage of proinflammatory cytosolic proteins, the potentiation of macrophage secretion, and the bystander effect of inflammation.

Neutrophil Apoptosis Is Delayed in Sepsis

Neutrophil apoptosis is delayed in neutrophils exposed to superantigen, in patients suffering from SIRS, in trauma patients with septic complications, and in ICU patients with severe sepsis (43–46). There is evidence that the signal which delays neutrophil apoptosis is a soluble mediator. For example, the supernatant of cultured neutrophils exposed to superantigen can delay normal neutrophil apoptosis (47). Serum from patients suffering from SIRS and sepsis also delayed apoptosis in neutrophils from healthy controls (43–45). Interestingly, serum from trauma patients suffering from septic complications could delay neutrophil apoptosis in a dose-dependent manner (45).

Blocking antibody to GM-CSF in media stimulated with superantigen, staphylococcus enterotoxin B, and in serum from patients with SIRS restored normal neutrophil apoptosis (44, 47). In another study, anti-G-CSF, but not anti-GM-CSF abrogated the effects of serum from trauma patients with septic complications (45). Antibody to TNF- α has been shown to restore a normal time course of apoptosis in neutrophils from ICU pa-

tients admitted for sepsis (46). Supplementation of interleukin (IL)-10 independently restored neutrophil apoptosis (44). Finally, the study looking at neutrophil apoptosis with superantigen also showed that γ IFN-blocking antibody could restore neutrophil apoptosis (46, 47). These data suggest that multiple soluble proinflammatory signals can influence the innate apoptotic machinery of the neutrophil and that the relative importance of each mediator may depend on the intensity of the injury stimulus, the type of antigen or species variability. Corroborative evidence implicates the degree of host injury to the amount of dysregulation of neutrophil apoptosis. For example, patients suffering from trauma and after a major operation did not have delays in neutrophil apoptosis. However, trauma patients with septic complications did have significant delays in neutrophil apoptosis (45).

In vivo studies have provided some insight but have failed to provide a conclusive picture. Ayala and colleagues (48), in a mouse model of polymicrobial sepsis, showed increased apoptosis in peritoneal granulocytes and decreased apoptosis in blood leukocytes. Anti-TNF- α binding protein decreased neutrophil apoptosis. This is in direct contrast to *in vitro* work presented above. Cox (49) looked at an *in vivo* model of lung inflammation by direct tracheal instillation of LPS. He noted that IL-10, an anti-inflammatory, proapoptotic cytokine significantly decreased the time to resolution of neutrophilia. *Ex vivo* culture showed that neutrophils were phagocytosed by macrophages.

The unifying theme points to the observation that signals which up-regulate inflammation tend to delay neutrophil apoptosis. The degree of inflammation appears to be important quantitatively in the degree of impairment seen. Different models highlight the impact of different cytokines. Blocking some of these mediators can restore the normal time course of neutrophil apoptosis in some, but not all, models of sepsis. *In vivo* studies show some disparity in the findings seen *in vitro*. This underscores the redundancy of the immune system in physiologic conditions. The key, irreplaceable and/or irrevocable factors involved in neutrophil apoptosis are not known. More importantly, they may not exist. Interdependent back-up mechanisms may be able to sufficiently initiate appropriate injury signals to the neutrophil. Artificial models of sepsis and inflammation may be

emphasizing a particular pathway in a situation that may not be physiologically relevant. These findings are reminiscent of the pleiotropic effects of proinflammatory cytokines and the tangled web of cytokine physiology from the previous decade. The available data, however, fall well short of linking the finding of delays in neutrophil apoptosis with a change in pathophysiologic outcome. It is not clear from any of these studies, for example, whether delaying apoptosis in neutrophils is beneficial or detrimental. Delay in apoptosis may be a marker of severe inflammation. Tying a pathophysiologic role to this finding will require focused, experimental manipulation of neutrophil apoptosis *in vivo* linked to a change in clinical outcome.

LYMPHOCYTES

Apoptosis plays a role in the termination of lymphocyte responses and in the deletion of autoreactive lymphocytes (50, 51). Lymphocytes require two signals for activation, terminal differentiation and proliferation. The first signal is antigen which accounts for the specificity of lymphocyte responses. The second signals are either from costimulatory molecules on antigen-presenting cells or cytokines. The best categorized costimulatory molecules are the B7 family, B7-1 (CD80), and B7-2 (CD86), which are up-regulated on activated professional antigen-presenting cells like dendritic cells. These molecules interact with CD28 on T lymphocytes and induce transcription of the lymphocyte growth factor, IL-2, and the anti-apoptotic protein, Bcl-xL. Failure of an appropriate second signal after antigen stimulation results in anergy (clonal unresponsiveness) or apoptosis (52).

With respect to termination of antigen-induced lymphocyte responses, apoptosis is involved with each step of two signal activation scheme. Once the specific immune response is up-regulated, antigen is cleared from the system. Antigen reactive clones of necessity receive fewer "first" signals. While some of these clones will transform into memory cell and perform surveillance function, the majority of these clones will undergo apoptosis (53). The signals which regulate this transformation are not well understood. With respect to costimulatory "second" signals, upon T-cell activation, a number of inhibitory pathways are initiated. Antigen stimulation results in the up-regulation of the cell surface molecule

CTLA4 on the lymphocyte, which tightly binds to B7 molecules. CTLA4, although variably expressed in frequency and intensity, have the exact opposite effects of the activating molecule CD28. Ligation of CTLA4 results in inhibition of IL-2 secretion, cell cycle arrest, and apoptosis (54).

Apoptosis also plays an important role in tolerance to autologous antigen. For example, antigen-presenting cells are continuously ingesting, processing, and presenting self and nonself protein. Only foreign antigens with the appropriate cytokine stimulation can generally up-regulate the full complement of costimulatory molecules. Therefore, T lymphocytes which autoreact to self-antigen do not induce autoimmune responses because they lack appropriate or sufficient costimulation. Second, activation of the T cell results in the simultaneous expression of Fas, a member of the TNF receptor family of death receptors. Full expression of Fas requires repeated T-cell receptor stimulation. This may point to another tolerance mechanism. Self-antigens, which are more likely to cause repeated T-cell receptor ligation, may induce Fas expression and prime autoreactive lymphocytes for active cell death (55). Finally, lymphocyte stimulation induces a negative feedback effect with IL-2. The molecular mechanisms underlying this effect are not well understood, but targeted deletion of IL-2 gene or its receptor does not result in the expected immunodeficiency. Rather, the phenotype which is seen is one of uncontrolled lymphocyte proliferation and autoimmune reactions (56, 57).

Lymphocyte Apoptosis is Increased in Sepsis

In vivo studies have identified that lymphocyte apoptosis is increased in models of experimental polymicrobial bacterial peritonitis (cecal ligation and puncture [CLP]). Increases in apoptosis have been reported in thymocytes, bone marrow-derived B lymphocytes, intraepithelial lymphocytes in the gut, lamina propria lymphocytes, Peyer's patch B lymphocytes, and splenocytes (58–62).

Using targeted gene knockout strains of mice, it has been observed that subpopulations of lymphocyte are sensitive to different inducers of apoptosis. Apoptosis in thymocytes, splenocytes, and bone marrow-derived cells has been shown to not be differentially dependent on lipopolysaccharide (LPS) or TNF- α

(59). Intestinal epithelial cells and Peyer's patch B lymphocytes, however, show increased apoptosis in endotoxin-resistant animals, but no increases in Fas ligand deficient strains (60, 61). This implies an LPS-independent and TNF- α dependent signaling pathway. This difference has been hypothesized to be a consequence of repeated exposure of intestinal lymphocytes to intraluminal antigens.

Rag knockout strains have a deletion of the recombinase that is necessary for immunoglobulin and TCR expression. These mice do not have mature lymphocytes and exhibit an immunodeficient phenotype. In response to CLP, increased apoptosis is seen in lymphoid and non-lymphoid organs, suggesting that apoptosis is not dependent on mature lymphocytes (63). Outcome from sepsis in this group is controversial. One study reported a decrease in survival, but another report from the same group noted no premature death in the Rag knockouts (63, 64).

Expression of the anti-apoptotic protein bcl-2 has been shown to be decreased in a model of sepsis by laparotomy and LPS injection in pigs (65). Transgenic mice which overexpress bcl-2 in T lymphocytes have decreased apoptosis in both T cells and in splenic B lymphocytes, indicating a potential bystander effect. *Ex vivo* culture of Bcl2 overexpressing lymphocytes showed no loss in mitochondrial membrane potential compared with control lymphocytes. Interestingly, Bcl2 overexpressing mice have increased survival after experimental polymicrobial peritonitis (64).

Treatment of animals with steroid antagonists has shown that apoptosis in thymocytes, but not bone marrow-derived lymphocytes, is dependent on endogenous steroid, but not LPS or TNF- α . Interestingly, treatment with steroid antagonist showed an increase in bcl-2 and Fas expression (58).

Taken together, it appears that lymphocyte apoptosis is sensitive to the effects of physiologically relevant models of bacterial sepsis. A continuing theme is that subpopulations of cell types are differentially sensitive to different initiators of apoptosis. The TNF- α transduction pathway may be more critical in inducing some subpopulations of lymphocytes. Additionally, the anti-apoptotic protein bcl-2 has a protective effect in several models of lymphocyte apoptosis. Improved survival with bcl-2 overexpression implies that premature, increased, or ac-

celerated lymphocyte apoptosis may be detrimental. One possible hypothesis is that increased lymphocyte apoptosis decreases the ability to up-regulate a specific immune response to rapidly and effectively clear infection which may result in the compensatory up-regulation of nonspecific, and inherently less-efficient mechanisms of host defense. Alternatively, however, premature lymphocyte apoptosis may simply be a marker of a severe inflammation. The finding of improved survival with decreased lymphocyte apoptosis, however, gives one of our earliest clues in linking changes in apoptosis with clinical outcome.

END-ORGAN APOPTOSIS IN SEPSIS

In vivo studies have identified that parenchymal tissue undergoes increased apoptosis in models of sepsis. In response to intravenous injection of LPS, Bohlinger et al. (66) noted apoptosis in the liver, lung, kidney and intestine of mice. They noted the degree of apoptosis correlated with serum TNF- α levels and that apoptosis was attenuated by endogenous release of nitric oxide. Hiramatsu et al. (67), in a model of polymicrobial peritonitis, noted that apoptosis was increased in ileum, colon, lung, kidney, and skeletal muscle. Using Rag knockout mice, they observed a similar distribution and degree of apoptosis in comparison with wild type mice and concluded that mature lymphocytes were not required for apoptosis and that apoptosis occurred in nonlymphoid cells. In a follow-up study of rapid autopsy analysis in septic human patients, Hotchkiss et al. (68) noted focal apoptosis in the spleen, colon, and ileum. They noted a depletion of lymphocytes in the white pulp of spleens with a concomitant lymphocytopenia in 79% of septic patients, but not in any nonseptic patients. Detection of cleaved caspase was significantly increased in the spleens of septic vs. nonseptic patients. They proposed that apoptosis contributed to a lymphopenic and potentially immunosuppressed state.

Endothelial Cell Apoptosis in Sepsis

Endothelial dysfunction and damage are thought to contribute to organ dysfunction in sepsis. High levels of TNF- α or circulating LPS may possibly lead to endothelial cell (EC) apoptosis in endo-

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toxemia. Injection of LPS and TNF- α , for example, leads to ceramide-dependent endothelial apoptosis in mice (69). Drab-Weiss et al. (70) have noted that the antioxidant properties of amino thiols protected against LPS-induced EC apoptosis. DeMeester et al. (71) have noted that EC apoptosis in response to LPS is decreased with nitric oxide (NO) donors in a cGMP-independent fashion. They also noted that EC apoptosis correlated with NF κ B activity and that the preinduction of the heat shock response decreased NF κ B activity and EC apoptosis in response to LPS. Hu et al. (73) noted in human ECs, however, that there is an up-regulation of cytoprotective proteins A1 and A20 in response to LPS and that this up-regulation is dependent on NF κ B activity. We (74) have shown that adenoviral transduction of the inducible NO synthase (iNOS) gene into sheep ECs protected against LPS-induced apoptosis. Subsequent work noted that NO inhibited LPS-induced activation of caspase 3-like activity in a dithiothreitol sensitive fashion and S-nitrosylation of caspase 3 in TNF- α -treated ECs (75, 76).

It appears that EC apoptosis may be mediated by an LPS-induced oxidant injury and that regulation of the cytokine transcription signal, NF κ B, may be involved. There is some evidence to suggest that protective mechanisms may include the up-regulation of proteins. It appears that NO serves a protective role in EC function which is consistent with its known biological roles in vascular homeostasis although the physiologically relevant molecular mechanisms and pathways are still being worked out.

Hepatocyte Apoptosis in Sepsis

A recurring theme is that different cell populations, subtypes, and organs have a differential sensitivity to injury stimuli. The liver exhibits a number of cytoprotective mechanisms including some which limit apoptosis. This probably explains the limited apoptosis observed in the liver in models of sepsis. Whether activation of apoptotic signaling pathways contribute to dysfunction of organs such as the liver is unknown. One protective pathway involved in the liver is inducible NO synthase (iNOS). Ou et al. (77) blocked NO production in an *in vivo* perfusion of rat livers with selective inhibitors (which blocks iNOS) and nonselective inhibitors (which block iNOS and the constitutive isoform of NO synthase,

eNOS) via the portal vein. They saw that nonspecific inhibition of NO synthase resulted in severe hepatic necrosis and hepatocyte apoptosis. Infusion of inhibitors specific to the iNOS isoform resulted in hepatocyte apoptosis only. The effect was partially abrogated by the infusion of an NO donor which localizes in the liver (77). These results suggest that the constitutive isoform of NO synthase was important in preventing necrosis possibly by maintaining the integrity of hepatic microcirculation while the inducible isoform was protective against hepatocyte apoptosis. NO appears to inhibit hepatocyte apoptosis by two mechanisms (78). First, NO stimulates cGMP production which blocks caspase activation and cytochrome *c* release from mitochondria. Second, NO can directly inhibit caspase activity by a process known as S-nitrosylation (78–81). This process is very efficient in hepatocytes and also endothelial cells and may explain the capacity of NO to protect these cell types, but not others. Whether organ failure can result from increased parenchymal apoptosis is unknown. It is reasonable to speculate that excessive initiation of the apoptotic signaling pathway, whether fully executed or not, could interfere with cellular and whole organ function.

OUTCOMES

Apoptosis occurs in sepsis. We have seen that it effects the nonspecific, innate as well as the antigen specific, learned mechanisms of the immune response. We have seen that apoptosis also occurs in parenchymal cells in models of sepsis. Finally, we have seen how infectious/proinflammatory stimuli can affect the apoptotic mechanisms in a variety of cell types. This has been instrumental in delineating the intricate signaling pathways in the apoptotic machinery. The impact of apoptosis on immunocompetence, organ function, and outcome has not been well detailed. There have, however, been a few studies which have indirectly implied a role for apoptosis in the pathophysiology of sepsis.

Ford et al. (82) analyzed specimens from 15 patients undergoing resection for necrotizing enterocolitis (NEC) and compared them with specimens from patients undergoing resection for other conditions including ileal atresia, peritonitis, intussusception, or cecal perforation. They noted that there was a significant and large increase in apoptosis at

the tips of villi and that this localized to the enterocyte in NEC patients. They also noted an up-regulation in iNOS in all NEC patients, but in no control patients. They postulated that the increase in apoptosis contributed to gut barrier failure.

Rats exposed to Zn²⁺ before a lethal dose of LPS showed induction of heat shock proteins, decreased apoptosis in lung, liver, and kidney, and improved survival (83). Mice with lymphocytes which overexpress Bcl-2 exhibited decreased lymphocyte apoptosis, preservation of mitochondrial membrane potential and improved survival after polymicrobial sepsis. This implies that lymphocyte apoptosis may have a detrimental effect, possibly by causing immunosuppression (64). However, mice in which the iNOS gene has been removed showed decreased survival, and decreased thymocyte apoptosis (84). This demonstrates a beneficial effect of iNOS and increased thymocyte apoptosis on survival. This implies that the effects of apoptosis on subpopulations lymphocytes have different roles in sepsis. The pathophysiologic role of apoptosis and its regulation are still being worked out.

SUMMARY

The need to maintain homeostasis requires compensatory up-regulating and down-regulating mechanisms to maintain the internal milieu. Apoptosis is one of the mechanisms that multicellular organisms have evolved as a global down-regulator of processes. It is seen in situations when the organisms must rid itself of cells which are no longer needed, that have been produced in excess, that have developed improperly, or that have sustained irreparable damage. In contrast to necrosis, apoptosis is a mode of cell death which is tightly regulated, energy dependent, and noninflammatory. It well serves the purpose of controlled elimination of extra cells. Apoptosis is critical in the normal control of leukocytes, which are continuously being generated at a basal rate, which must be up-regulated in response to infection, and which subsequently must be down-regulated once the infectious stimulus has been eliminated.

Sepsis marks a pathologic dysregulation of the immune system in response to infection. It is seen as an uncontrolled upregulation of proinflammatory mediators which ultimately results in multiple organ failure. Recent work has looked into the possibility that dysregulation of

down-regulating mechanisms of inflammation may be responsible for the persistence of hyperinflammation.

It can be seen that sepsis is associated with changes in the dynamics and regulation of apoptosis in comparison with the uninfected state. These changes are seen not only in nonspecific mediators of host defense, like neutrophils, but also in the specific, responsive arm of the immune system, lymphocytes. It has also been seen that changes in apoptosis can be altered by blocking antibody to proinflammatory mediators, steroid antagonists, and by transfection of anti-apoptotic molecules. Some of these manipulations have shown changes in outcome, but have not consistently demonstrated a clear change in the pathophysiology of this very complex immune state. In other words, it is still not clear whether delayed neutrophil apoptosis or increased lymphocyte apoptosis is bad or good.

Previous clinical trials that have tried to manipulate the host inflammatory response in SIRS and sepsis have failed to show benefit in blocking proinflammatory cytokines. One of the reasons that these experiments have not shown a beneficial effect may be due to the complexity and diversity of host defense. The immune response has evolved multiple back-up mechanisms, and a stimulus can often set off a cascade of events down separate pathways. Manipulation of any one pathway down stream from the central stimulus can have a multitude of potential effects which are difficult to predict in the clinical situation if extrapolated even from well designed experimental models. Future work in the study of the impact of apoptosis in sepsis must account for functional outcomes to give context and relevance in applicable situations.

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